Biochimica et Biophysica Acta 1851 (2015) 929–936

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

c-Flip KO fibroblasts display lipid accumulation associated with endoplasmic reticulum stress

Claudia Giampietri [a,*,1], Simonetta Petrungaro [a,1], Silvia Conti [a], Antonio Facchiano [b], Antonio Filippini [a], Elio Ziparo [a]

[a] Istituto Pasteur-Fondazione Cenci Bolognetti, Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Section of Histology and Medical Embryology, Sapienza University of Rome, Rome, Italy

[b] Istituto Dermopatico dell'Immacolata IDI–IRCCS, Rome, Italy

ARTICLE INFO

Article history:
Received 24 October 2014
Received in revised form 4 February 2015
Accepted 26 February 2015
Available online 5 March 2015

Keywords:
Mouse embryonic fibroblasts
Lipid droplets
Autophagy
ER stress
c-Flip

ABSTRACT

c-Flip proteins are well-known apoptosis modulators. They generally contribute to tissue homeostasis maintenance by inhibiting death-receptor-mediated cell death.

In the present manuscript, we show that c-Flip knock-out (KO) mouse embryonic fibroblasts (MEFs) kept in culture under starvation conditions gradually modify their phenotype and accumulate vacuoles, becoming progressively larger according to the duration of starvation. Large vacuoles are present in KO MEFs though not in WT MEFs, and are Oil Red-O positive, which indicates that they represent lipid droplets. Western blot experiments reveal that, unlike WT MEFs, KO MEFs express high levels of the lipogenic transcription factor PPAR-γ. Lipid droplet accumulation was found to be associated with endoplasmic reticulum (ER) stress activation and autophagic modulation valuated by means of BIP increase, LC3 lipidation and AMP-activated protein kinase (AMPK) phosphorylation, and p62 accumulation. Interestingly, XBP-1, an ER stress-induced lipogenic transcription factor, was found to preferentially localize in the nucleus rather than in the cytoplasm of KO MEFs.

These data demonstrate that, upon starvation, c-Flip affects lipid accumulation, ER stress and autophagy, thereby pointing to an important role of c-Flip in the adaptive response and ER stress response programs under both normal and pathological conditions.

1. Introduction

Environmental stresses that threaten cell homeostasis trigger various cellular responses ranging from survival pathway activation to apoptosis. The response to cellular stress depends to a large extent on the nature and level of the insult as well as on the cell type. The interplay between all stress responses ultimately determines the fate of the stressed cell. The proteins that control stress responses include cellular FLICE-inhibitory (c-Flip) proteins, which play an important role by protecting cells from both extrinsic and intrinsic programmed cell death. In mammalian cells three splice variants are expressed: c-Flip$_L$, c-Flip$_S$, and c-Flip$_R$ [9]. Flip$_L$ is structurally similar to caspase-8 since it contains two tandem death effector domains (DEDs) and an inactive C-terminal caspase-like domain. c-Flip$_S$ and c-Flip$_R$ have the same DEDs as Flip$_L$ but have shorter C-terminal sequences. Although all c-Flip isoforms efficiently inhibit caspase-8 activation, the role of c-Flip$_L$ in the death-inducing signaling complex (DISC) is controversial. Indeed, some reports indicate that c-Flip$_L$ is an anti-apoptotic molecule that functions similarly to c-Flip$_S$, whereas others describe c-Flip$_L$ as a pro-apoptotic molecule, demonstrating its role in the autocatalytic activation of procaspase-8 at the DISC ([3,7]; Giampietri et al., 2006;[6,8,24]).

c-Flip proteins represent key players of stress response mechanisms. For instance, various types of stressing conditions down-regulate c-Flip proteins, thereby sensitizing cells to programmed cell death. Oxidative stress sensitizes bladder cancer cells to TRAIL-mediated apoptosis by down-regulating various anti-apoptotic proteins, including c-Flip proteins [40]. Heat stress also down-regulates c-Flip, thereby sensitizing cells to Fas receptor-mediated apoptosis and promoting caspase-8 cleavage [38].

Furthermore, in response to environmental and physiological stress conditions that increase endoplasmic reticulum (ER) stress, i.e. the load of unfolded proteins in the ER [20], cells are sensitized to TRAIL-induced apoptosis by c-Flip down-regulation [23]. More recently, TRAIL-induced death in melanoma cells has been directly related to c-Flip expression, further suggesting that these proteins play a key role in cancer cell death [39].

* Corresponding author at: Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Section of Histology and Medical Embryology, "Sapienza" University of Rome, Via A. Scarpa, 14 00161 Rome, Italy. Tel.: +39 6 49766948; fax: +39 06 4462854.
E-mail address: claudia.giampietri@uniroma1.it (C. Giampietri).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbalip.2015.02.018

Various types of cellular stress have been shown to induce lipid droplet (LD) biogenesis in mouse tissues. LDs store neutral lipids, including triacylglycerol and cholesterol esters [10], and are surrounded by a layer of amphipathic lipids, such as phospholipids and cholesterol, and proteins [22]. LDs accumulate within the cell in response to exogenous lipid availability and as a consequence of different kinds of cellular stress, including inflammation and oxidative stress. Although fatty acids contained in LDs are hypothesized to play a protective role against stressors [17], the molecular mechanisms underlying fatty acid uptake and biosynthesis under stress conditions are largely unknown. ER stress is one of the cellular stresses reported to induce LD accumulation [15]. ER stress occurs when unfolded or misfolded proteins accumulate in the lumen of the endoplasmic reticulum. This condition activates different signaling pathways to resolve the cellular stress by regulating processes on either side of the ER membrane through an adaptive mechanism termed the unfolded protein response (UPR). The UPR allows a cell to increase the folding capacity of the ER. ER stress signaling is mediated by three proximal sensors, i.e. PERK, the IRE1 (inositol-requiring protein 1α)/XBP-1 (X-box binding protein 1) system and ATF6 (activating transcription factor 6), all of which are ER transmembrane receptors that constantly monitor the ER state. Under normal conditions, each receptor is maintained inactive through binding, via its luminal domain, with the ER chaperone protein BIP. Accumulation of unfolded proteins triggers dissociation of BIP from each receptor, thereby facilitating its activation and the up-regulation of BIP synthesis [18]. Initial signaling from each stress receptor is aimed at reducing levels of unfolded proteins and restoring cellular homeostasis. However, sustained or excessive ER stress results in a switch from survival to death signaling. IRE1 is one of the three branches of the UPR. IRE1 contains a kinase domain and exerts endoribonuclease activity. Dimerization and autophosphorylation of IRE1 triggers endonuclease activity, which induces alternative splicing of XBP-1 mRNA and expression of the active XBP-1 transcription factor [28]. The XBP-1 transcription factor translocates into the nucleus and activates the transcription of genes such as those coding for ER chaperones and lipid biogenesis enzymes [42]. The ER stress activation associated with increased XBP-1 levels has also been shown to mediate the Vemurafenib pro-apoptotic effect in melanoma cells [2].

In a murine knock-out (KO) model, the essential role of Flip proteins was shown to inhibit death receptor-mediated apoptosis induced by Fas or TNF-R1 engagement [13]. Embryonic fibroblasts isolated from a Flip −/− murine model have previously been characterized in terms of their apoptosis sensitivity [13] and mitochondria-endoplasmic reticulum exchanges [21]. In the present manuscript, we show that c-Flip −/− mouse embryonic fibroblasts (MEFs) display an unexpected ability to survive in long-term cultures in the absence of regular medium changes or supplementation, and we characterize their phenotype. We show that c-Flip −/− MEF resistance correlates with LD accumulation in the presence of XBP-1 lipogenic transcription factor nuclear translocation, thereby demonstrating a novel role for c-Flip proteins in stress responses.

2. Materials and methods

2.1. Cell cultures and reagents

Wild type (WT) and c-Flip −/− MEFs were a generous gift from Tak W. Mak (Amgen Institute, Toronto, Canada).

MEFs were cultured in DMEM, enriched with 10% fetal bovine serum, glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), and non-essential amino acids, in the presence of penicillin (100 U/ml) and streptomycin (100 μg/mL). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. MEFs were plated in 35 mm dishes and grown for the number of days indicated in complete medium without medium changes. Bafilomycin A1 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cells were treated with 1 nm Bafilomycin A1. The PPAR-γ antagonist, referred to as GW9662, was purchased from Cayman chemical (USA). Cells were treated for 4 days with 10 μM GW9662 or with the vehicle DMSO. Cells were photographed on the selected days of culture by microscopy (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.). Images were obtained at room temperature using an AxioCamHRC camera (Carl Zeiss Microimaging, Inc., Milan, Italy) by Axiovision 3.1 software and images were assembled in panels using Photoshop 7.0 (Adobe, Waltham, MA, USA).

2.2. Immunoblotting

Cells were harvested and lysed in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) in the presence of a complete protease-inhibitor mixture (Sigma-Aldrich). The protein concentration was determined by micro BCA assay (Pierce, Rockford, IL, USA) and the proteins were separated by SDS–PAGE and transferred on nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ). Membranes were probed using the following antibodies: anti-β-actin, anti-Histone H-3 (Sigma-Aldrich), anti-PPAR-γ, anti-P-AMPK, anti-LC3 (Cell Signaling, Danvers, MA), anti-p62 (Abcam, Cambridge, UK), anti-XBP-1 (M186; Santa Cruz Biotechnology, INC, CA), and anti-BIP/GRP78 (BD Transduction Laboratories, USA). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Biorad, USA). Membranes were washed with Tris-buffered saline with 0.1% Tween-20 and developed using the chemiluminescence system (ECL Advance; Amersham Bioscience, Piscataway, NJ).

2.3. Nuclear extract preparations

Proteins were extracted on ice and cells were harvested by scraping with 1 mL of ice cold PBS, then centrifuged for 5 min at 1500 rpm. The pellet was re-suspended in equal volumes of ice cold hypotonic buffer (10 mM Tris pH 7.5, 1.0 mM PMSF). A loose pestle was used with a Wheaton Dounce Tissue Grinder (Fisher Scientific) to lyse cells. Trypan blue was used to verify that cells were lysed before samples were centrifuged for 7 min at 900 *g*. Supernatant contained cytosolic proteins that were then stored at −80 °C. The nuclear pellet was re-suspended in SDS 20×.

2.4. Oil Red-O staining of lipid droplets

On the selected days of culture, MEF monolayers were washed with PBS and then fixed with 10% formalin for 5 min. Cells were washed with 60% isopropanol for 5 min, then left to dry completely. A 0.5% Oil Red-O/isopropyl alcohol solution was added for 1 h to the cells, which were then washed several times with distilled water and tap water. The stained cytoplasmic lipids were visualized and photographed by microscope (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.) with ×10 NA 0.30, ×20 NA 0.50, ×40 NA 0.75 or ×63 NA 1.25 oil objective lenses (Plan-Neofluar). Images were obtained at room temperature using an AxioCamHRC camera. Lastly, the stained cells were destained with isopropanol and the Optical Density (OD) of the destaining isopropanol was measured by spectrophotometry at a 510 nm wavelength.

2.5. Immunofluorescence

Immunofluorescence was performed as previously described [5]. Briefly, the cells were fixed with 4% paraformaldehyde (Sigma) and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The samples were then blocked with 5% goat serum for 30 min and incubated overnight with the rabbit anti-XBP-1 or anti-calnexin. Following washes with PBS, secondary goat anti-rabbit IgG-FITC (1:200 Sigma) was added to the samples for 1 h. The cells were then counterstained with Hoechst (Sigma) in calnexin immunofluorescence experiments. Immunofluorescence was visualized by a microscope (Axioskop 2 plus; Carl Zeiss Microimaging, Inc.). Images were obtained at room temperature

using an AxioCamHRC camera (Carl Zeiss Microimaging, Inc., Milan, Italy) and Axiovision 3.1 software.

2.6. Statistical analysis

Values are expressed as mean ± standard error (s.e.m.). The statistical analyses were performed by student's *t* test; a P value < 0.05 was considered statistically significant.

In the AMPK experiments, correlation between AMPK activation and number of days in culture was investigated by Pearson's correlation with GraphPad Prism software version 5.04 (GraphPad Prism software Inc.).

3. Results

3.1. Lipids accumulate in c-Flip knock-out mouse embryonic fibroblasts

c-Flip KO MEFs have been described previously [13]. We confirmed c-Flip protein ablation and higher apoptotic sensitivity in KO MEFs than in WT MEFs following TNF-alpha stimulation, as previously reported [13] (data not shown). Under basal culture conditions and with no changes in medium, after reaching confluence WT MEFs start to detach from the plate and many cells die (Fig. 1A panel b). By contrast, in the cytoplasm of c-Flip KO MEFs, vacuoles begin to appear and their number and size gradually increase over time (Fig. 1A panels d and e). Around day 40, very large vacuoles are visible in KO plates, probably as a result

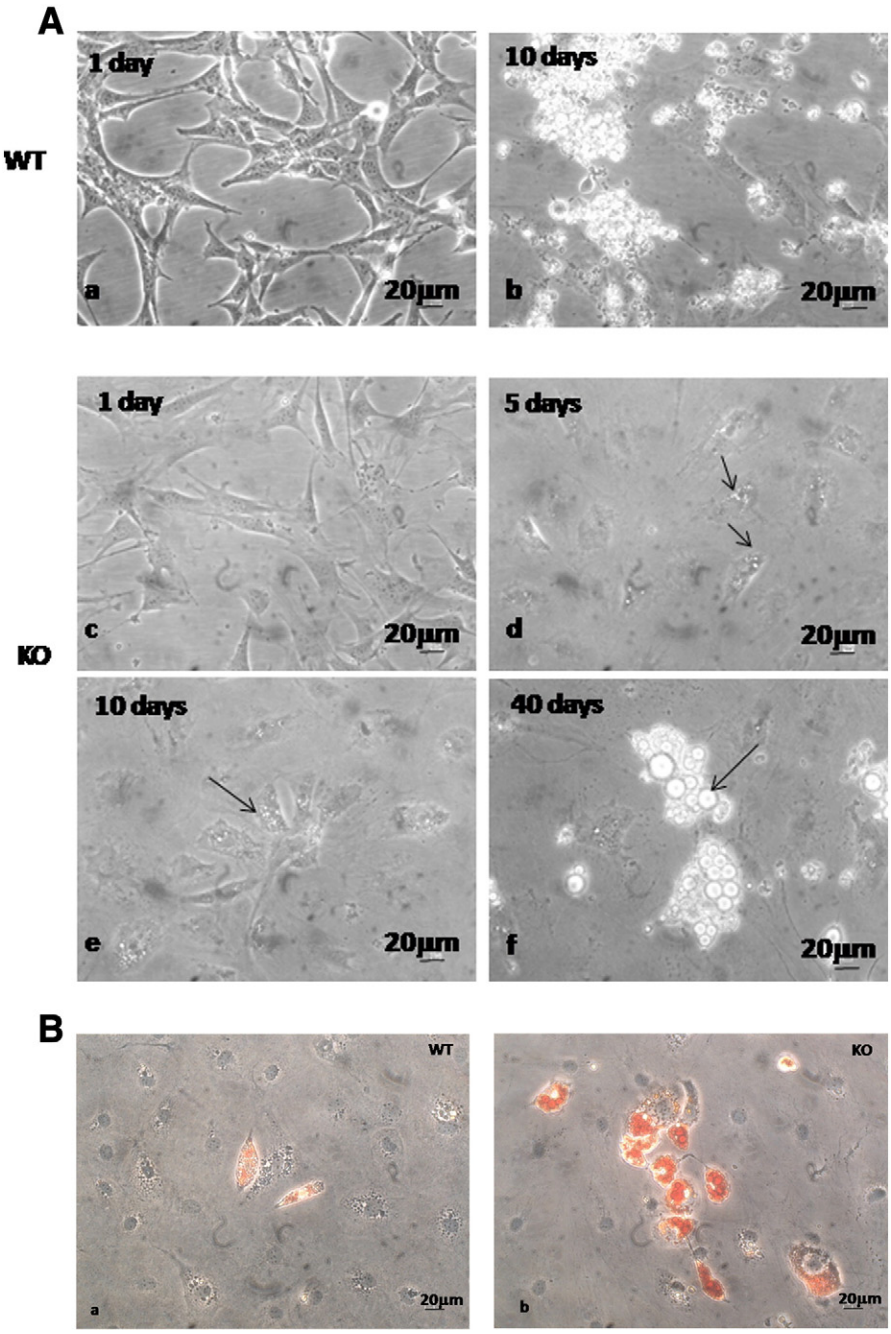

Fig. 1. Accumulation of lipid droplets in c-Flip KO MEFs. A) Bright-field microscopic images of cultured MEFs (both WT and KO) at different time points after plating (day 1: a, c; day 5: d; day 10: b, e; day 40: f). The pictures reveal gradual vacuole accumulation in KO cells. B) Oil Red-O staining. Cells were stained on day 7 of culture with Oil Red-O dyes as described in "Materials and methods". When photographed under the bright-field microscope, they revealed lipid droplet accumulation in KO cells. Black arrows indicate vacuoles.

of fusion events (Fig. 1A panel f). Oil Red-O dye staining experiments revealed an accumulation of triglycerides, which indicated that the vacuoles extensively observed in KO MEFs correspond to lipid droplets (Fig. 1B). To confirm the tendency displayed by KO MEFs to accumulate more triglycerides than WT MEFs, Oil Red-O staining was carried out on both WT and KO MEFs 3 days after plating when vacuoles are not yet visible; subsequently, the Oil Red-O in the stained cells was eluted with isopropanol and OD of the elution was assessed by spectrophotometry. Significantly higher OD values were measured in the isopropanol elution of Oil Red-O staining of KO cells than that of WT cells (0.22 ± 0.005 vs 0.17 ± 0.003 $P < 0.05$), thus indicating that KO MEFs have a higher lipid content. Upon brefeldin treatment, a widely used ER stress inducer, KO MEFs displayed stronger Oil Red-O staining than not-treated KO MEFs, thus suggesting that KO cells may accumulate lipids to a greater extent as a response to ER stress (data not shown).

3.2. Protein expression of the lipogenic transcription factor PPAR-γ in c-Flip KO MEFs

In order to shed light on the molecular mechanisms underlying lipid accumulation in c-Flip KO MEFs, Western blot experiments were carried out to analyze the transcription factor peroxisome proliferator-activated receptor-gamma (PPAR-γ). This is a well-known promoter of triglyceride synthesis and regulator of gene products involved in lipid storage [33]. We found that lipid accumulation was associated with high PPAR-γ protein expression in KO MEFs, while no expression was observed in WT (Fig. 2A).

We then assessed whether PPAR-γ plays a crucial role in lipid accumulation in KO MEFs by treating cells with the irreversible PPAR-γ antagonist 2-chloro-5-nitrobenzanilide GW9662. While higher mortality was observed in GW9662-treated KO cells, single-cell lipid droplet accumulation was drastically reduced if compared with untreated KO cultures (Fig. 2B).

3.3. Autophagosome increment in c-Flip KO MEFs correlates with LC3-II and AMPK activation but is not paralleled by complete autophagic degradation

To investigate the cellular mechanisms underlying lipid droplet accumulation in c-Flip KO MEFs, we first focused on autophagy. Autophagy is known to regulate intracellular lipid store [35]. Autophagy has also been involved in adipogenic differentiation of 3T3-L1 preadipocytes, in which it represses proteasome-dependent PPAR-γ2 degradation [41]. We therefore investigated LC3 lipidation in order to compare autophagy in KO and WT MEFs. While cells displayed comparable LC3-II levels one day after plating, LC3-II levels subsequently gradually increased in parallel with lipid droplet accumulation in KO cells though not in WT cells, thus pointing to autophagosome up-regulation in KO cells undergoing lipid storage increase (Fig. 3A). KO MEFs also displayed a significant and time-dependent increase in AMPK phosphorylation (Fig. 3B), whose role in autophagy induction through mammalian target of the rapamycin complex 1 (mTORC1) is well known [44].

We then investigated p62 expression, and found that the increase in LC3-II in KO cells at 40 days is paralleled by autophagy substrate p62 protein expression enhancement (Fig. 3C), which suggests that there is at least a partial blockage of autophagic cargo degradation. In order to better investigate the autophagic flux in KO MEFs, we treated cells with Bafilomycin A1, a well-known inhibitor of lysosomal degradation, and analyzed its effect on LC3-II turnover. While LC3-II levels increased in Bafilomycin A1-treated WT cells, as expected, they did not increase in Bafilomycin A1-treated KO cells, compared with untreated controls. This further suggests that autophagosome accumulation in KO cells may occur as a consequence of autophagic flux inhibition (Fig. 3D).

3.4. Lipid droplet accumulation parallels the increase in the ER stress marker

According to the results obtained, we hypothesized that lipid droplet accumulation might represent a stress response to long-term KO cell cultures. A hallmark of cells undergoing ER stress is ER expansion, which is designed to enhance the processing of the accumulated unfolded proteins [43]. Immunofluorescence staining achieved by an antibody to the ER protein calnexin revealed that the ER in KO cells is enlarged when compared with WT cells, as shown in Fig. 4. We therefore investigated whether KO MEFs that stockpile lipids display concomitant signs of ER stress induction.

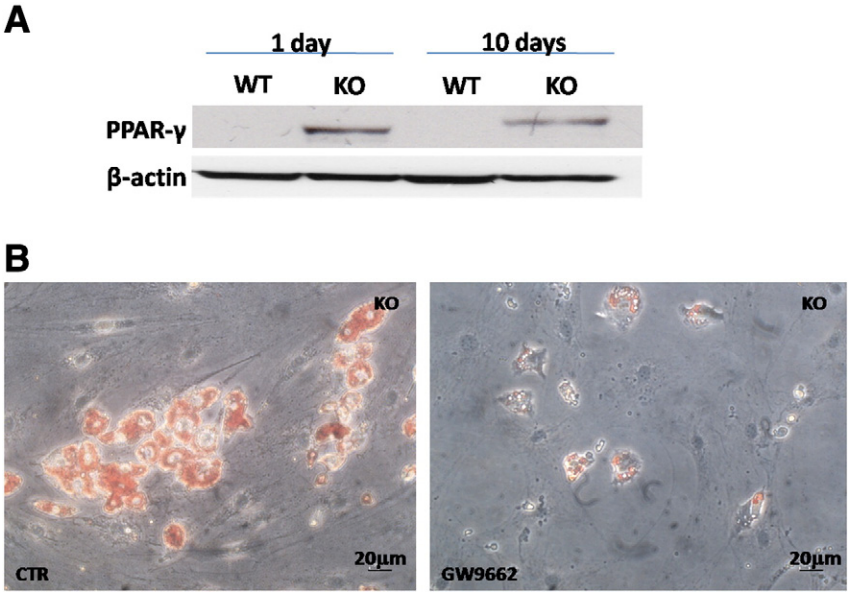

Fig. 2. PPAR-γ expression and function in WT and KO MEFs. A) Western blot analysis of PPAR-γ in WT and KO cells showing PPAR-γ expression in KO MEFs both on day 1 and day 10 of culture. β-Actin staining is shown for the loading control. Data shown are representative of three independent experiments. B) Oil Red-O staining of KO MEFs treated with the PPAR-γ inhibitor, compared with untreated cells, shows the inhibition of lipid droplet storage in the presence of the PPAR-γ inhibitor.

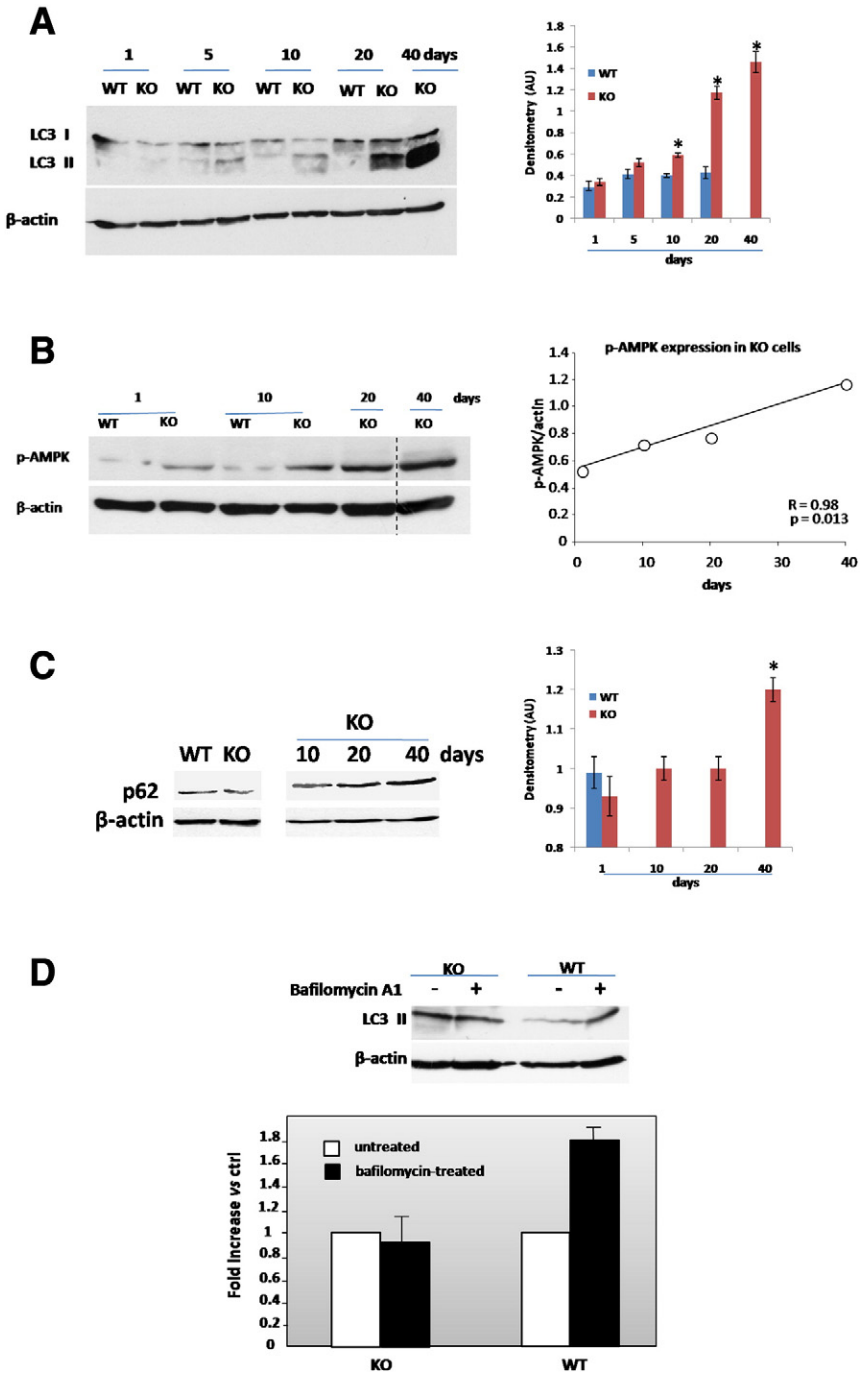

Fig. 3. Autophagy analysis in KO compared with WT MEFs over culture time. A) Western blot analysis of LC3 conversion in MEFs suggests significant autophagy activation in KO cells, compared with WT cells, after 10 days of culture. Densitometric analysis of LC3-II (a direct marker of autophagy activation) relative to β-Actin in three independent experiments is presented in the histogram as the mean ± s.e.m., expressed as arbitrary units. * Indicates $P < 0.05$ compared with the corresponding WT control. For the 40-day time point, the mean of all the WT measurements was considered. B) Western blot analysis of p-AMPK at different culture times. β-Actin staining is shown as a loading control. The graph shows the p-AMPK densitometry corrected according to β-Actin expression for each day of culture. A time-dependent increase in p-AMPK is observed in KO cells according to Pearson's correlation. One representative experiment of three is reported. C) Western blot analysis of p62 in WT and KO cells revealing p62 accumulation in KO MEFs over culture time. β-Actin staining is shown as the loading control. The histogram shows the p62 densitometric analysis relative to β-Actin in three independent experiments as the mean ± s.e.m., expressed as arbitrary units. * Indicates significant p62 accumulation after 40 days of culture compared with KO at T0 ($P < 0.05$). D) Western blot analysis of LC3-II in the presence or absence of Bafilomycin A1 in KO and WT MEFs on day 7 of culture. β-Actin staining is shown as the loading control. The graph reports average fold increase of LC3-II densitometry corrected according to β-Actin expression in Bafilomycin-treated cells compared with untreated cells in three independent experiments and indicates the block in autophagic flux in KO MEFs.

ER stress can be detected by monitoring the expression levels of ER stress marker genes, including BIP (lumenal binding protein). Surprisingly, we found that time-dependent lipid droplet accumulation (Fig. 1) correlates with a time-dependent increase in the ER stress-associated chaperone BIP in KO cells (Fig. 5), thus confirming ER stress induction in long-term KO cell cultures.

3.5. c-Flip KO MEFs display increased nuclear localization of the X-box-binding protein-1 (XBP-1) transcription factor

In order to investigate the molecular mechanisms underlying ER-stress-driven lipid accumulation in KO cells, we analyzed XBP-1 protein expression and subcellular localization. Unlike the unspliced XBP-1

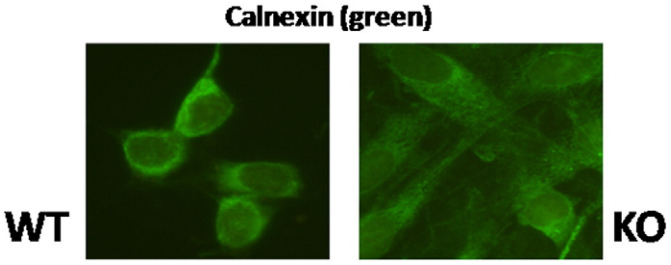

Fig. 4. Endoplasmic reticulum expansion in KO MEFs. Immunofluorescence staining of WT and KO MEFs using anti-calnexin antibody reveals strong ER expansion in KO compared with WT MEFs. Magnification × 400.

protein, which is rapidly degraded, nuclear spliced XBP-1 (XBP-1s) [37] leads to the expression of a number of UPR target genes, including those involved in lipid synthesis and ER biogenesis [1]. We analyzed XBP-1 levels in nuclear and whole-cell extract by Western blot in both WT and KO cells. We found higher level of XBP-1 in the nuclear extract of KO cells than in that of WT cells, whereas the level of XBP-1 in the overall cell extract of KO cells was comparable to that of WT cells (Fig. 6A). The higher XBP-1 nuclear localization we detected was confirmed by immunofluorescence in KO cells (Fig. 6B).

4. Discussion

It has been hypothesized that LDs are produced by the ER by means of particular, largely unknown, biogenesis events [29,30].

As LDs accumulate under various cellular stress conditions, their formation may to some extent be considered as a generalized response to stress. Indeed, it has been previously demonstrated that a close relationship exists between LD formation and the cellular response to ER stress. In particular, tunicamycin and brefeldin A, two agents that induce ER stress, have been shown to stimulate LD formation in *Saccharomyces cerevisiae* [4]. In mammals, LD accumulation is often induced by ER stress, regardless of increases in lipid concentrations within the cells [26,27]. LDs may play a role in ER stress response by allowing the retro-translocation of soluble misfolded proteins [11]. Glucose or nutrient deprivation, viral infections, lipid exposure, increased synthesis of secretory proteins and expression of mutant or misfolded proteins are among the conditions that trigger ER stress [14,19,32]. Under these conditions, cells activate the UPR. When chronic stress occurs (ranging from days to years), semi-permanent changes occur in cellular and ER functions. In such contexts, a relatively small number of cells may die, whereas the majority ultimately survive and adapt to the stressful stimulus. The central feature of an adaptive response to ER stress appears to be the persisting expression of proteins that facilitates survival, such as ER chaperones, which are not however accompanied by pro-apoptotic proteins (i.e. CHOP and GADD34). In particular, activation of ATF6α is believed to control the transcriptional regulation of various ER chaperones, including BIP, GRP94 and calreticulin [12,25].

In the present paper, we describe large-scale LD formation as a response to c-Flip ablation in embryonic fibroblasts during long-term culture in vitro in the absence of fresh medium supplementation. We observed that the accumulation of cytosolic LDs is associated with a UPR process (demonstrated by BIP accumulation and nuclear XBP-1 localization), which may represent a protective response in c-Flip −/− MEFs.

We further analyzed the c-Flip −/− phenotype and found PPAR-γ expression in KO MEFs though not in WT MEFs [16]. Since the selective PPAR-γ inhibitor GW9662 reversed lipid accumulation, we conclude that lipid accumulation in c-Flip −/− MEFs may be ascribed to a mechanism that is at least partially mediated by PPAR-γ. In this regard, it should be borne in mind that massive lipid droplet biogenesis under PPAR-γ control has recently been suggested to induce differentiation of cancer cells (including hepatocarcinoma, ovarian carcinoma and melanoma cells) toward adipocyte-like cells, thus highlighting a potential novel therapeutic strategy [31]. LDs accumulate within the c-Flip −/− MEFs cytoplasm, growing considerably larger in size. We analyzed the involvement of autophagy in this process because, besides contributing to the maintenance of the basal cellular metabolism, autophagy is also induced in response to various stresses, such as starvation, and is involved in lipid droplet accumulation [34].

We observed a time-dependent increase in both LC3 lipidation and AMPK activation in KO cells though not in WT cells. This finding points to a greater formation of autophagosomes in KO MEFs than in WT MEFs under starvation conditions. Nevertheless, we also observed in KO cells accumulation of p62, a marker of autophagic degradation. Since autophagy inhibition in cultured hepatocytes and mouse liver increased triglyceride storage in lipid droplets [35], we hypothesize that lipid accumulation may occur as a consequence of an impaired autophagic flux even in c-Flip −/− MEFs. The autophagic flux block in KO MEFs was confirmed by analyzing LC3-II turnover in the presence and absence of the lysosomal degradation inhibitor Bafilomycin A1.

In order to shed light on the molecular mechanisms underlying lipid storage in KO cells, we analyzed XBP-1, a potent UPR trans-activator that is known to be a positive lipogenic regulator and to play an important molecular role in acute ER stress-induced lipogenesis [36]. We found that XBP-1 nuclear localization was far more prevalent in KO cells than in WT cells, which leads us to speculate that XBP-1 controls events that determine lipid droplet accumulation following c-Flip ablation. Since active XBP-1, generated by UPR-mediated splicing of XBP-1

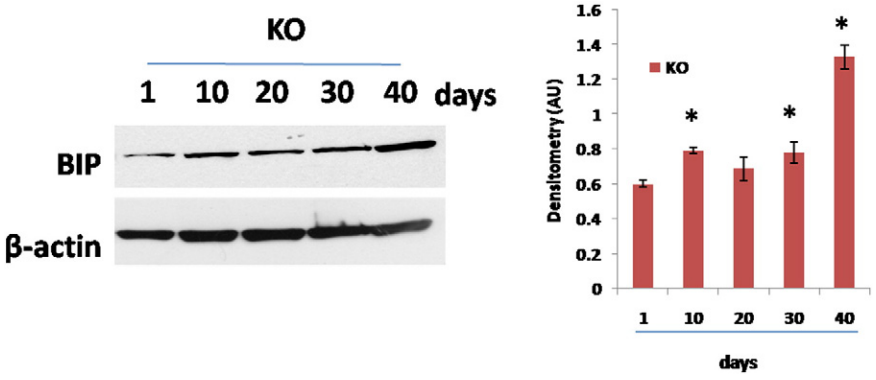

Fig. 5. ER stress activation in KO MEFs. Western blot analysis of BIP expression reveals gradual activation of the endoplasmic reticulum stress response over culture time in KO cells. β-Actin staining is shown as the loading control. The histogram shows the mean ± s.e.m. of the BIP densitometric analysis relative to β-Actin of three independent experiments, expressed as arbitrary units. * Indicates $P < 0.05$ compared with the day 1 time point.

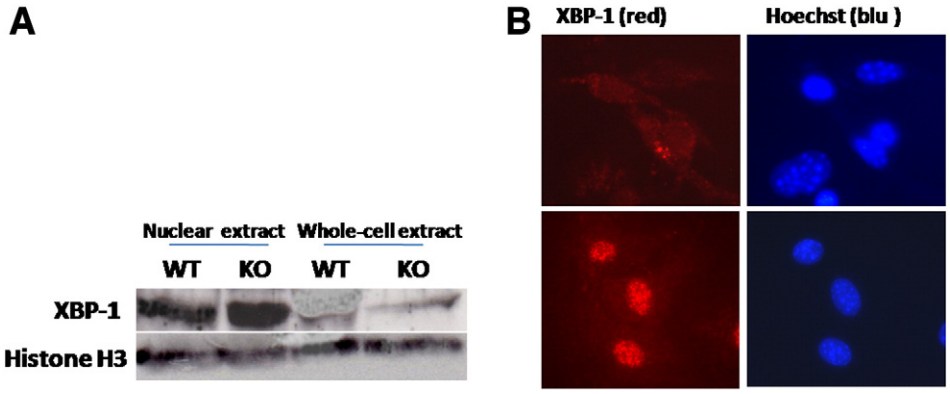

Fig. 6. XBP-1 nuclear localization in KO MEFs. Western blot analysis of XBP-1 expression reveals higher nuclear accumulation of XBP-1 in KO MEFs than in WT MEFs. Histone H3 staining is shown as the loading control. Data shown are representative of three independent experiments. B) XBP-1 immunofluorescence staining (red) revealing prevalent XBP-1 nuclear localization in KO compared with WT cells. The cells were counterstained with Hoechst to visualize nuclei. Magnification × 400.

mRNA, can dramatically enhance the expression of PPAR-γ in Huh-7 cells [16], we hypothesize that XBP-1 may also be responsible for PPAR-γ expression observed in KO cells.

To sum up, in the present manuscript we reveal a novel role played by the c-Flip protein in controlling cellular stress responses. We hypothesize that ER stress activation associated with c-Flip ablation might lead to lipid accumulation, a mechanism that ultimately allows cells to adapt to stress.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

We wish to thank Dr. Tak W. Mak for providing the WT and c-Flip −/− MEFs. We thank the researchers from the "P. Valdoni" Department of Sapienza University for their technical support.

This work was supported by PRIN 2010–2011, Fondazione Roma grants and "Ricerca Scientifica Sapienza 2014" to EZ.

The authors declare that they have no conflicts of interest.

References

[1] S. Basseri, R.C. Austin, Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential, Biochem. Res. Int. 2012 (2012) 841362.
[2] D. Beck, H. Niessner, K.S. Smalley, K. Flaherty, K.H. Paraiso, C. Busch, T. Sinnberg, S. Vasseur, J.L. Iovanna, S. Driessen, B. Stork, S. Wesselborg, M. Schaller, T. Biedermann, J. Bauer, K. Lasithiotakis, B. Weide, J. Eberle, B. Schittek, D. Schadendorf, C. Garbe, D. Kulms, F. Meier, Vemurafenib potently induces endoplasmic reticulum stress-mediated apoptosis in BRAFV600E melanoma cells, Sci. Signal. 6 (2013) ra7.
[3] D.W. Chang, Z. Xing, V.L. Capacio, M.E. Peter, X. Yang, Interdimer processing mechanism of procaspase-8 activation, EMBO J. 22 (2003) 4132–4142.
[4] W. Fei, H. Wang, X. Fu, C. Bielby, H. Yang, Conditions of endoplasmic reticulum stress stimulate lipid droplet formation in *Saccharomyces cerevisiae*, Biochem. J. 424 (2009) 61–67.
[5] C. Giampietri, S. Petrungaro, P. Coluccia, F. Antonangeli, K. Giannakakis, T. Faraggiana, A. Filippini, G. Cossu, E. Ziparo, c-Flip overexpression affects satellite cell proliferation and promotes skeletal muscle aging, Cell Death Dis. 1 (2010) e38.
[6] C. Giampietri, S. Petrungaro, P. Coluccia, F. Antonangeli, A. Paone, F. Padula, P. De Cesaris, E. Ziparo, A. Filippini, c-Flip(L) is expressed in undifferentiated mouse male germ cells, FEBS Lett. 580 (2006) 6109–6114.
[7] C. Giampietri, S. Petrungaro, P. Coluccia, A. D'Alessio, D. Starace, A. Riccioli, F. Padula, S.M. Srinivasula, E. Alnemri, F. Palombi, A. Filippini, E. Ziparo, P. De Cesaris, FLIP is expressed in mouse testis and protects germ cells from apoptosis, Cell Death Differ. 10 (2003) 175–184.
[8] C. Giampietri, S. Petrungaro, F.G. Klinger, P. Coluccia, A. Paone, E. Vivarelli, A. Filippini, P. De Cesaris, M. De Felici, E. Ziparo, c-Flip expression and function in fetal mouse gonocytes, FASEB J. 20 (2006) 124–126.
[9] A. Golks, D. Brenner, C. Fritsch, P.H. Krammer, I.N. Lavrik, c-FLIPR, a new regulator of death receptor-induced apoptosis, J. Biol. Chem. 280 (2005) 14507–14513.
[10] A.S. Greenberg, R.A. Coleman, F.B. Kraemer, J.L. McManaman, M.S. Obin, V. Puri, Q.W. Yan, H. Miyoshi, D.G. Mashek, The role of lipid droplets in metabolic disease in rodents and humans, J. Clin. Invest. 121 (2011) 2102–2110.
[11] I. Hapala, E. Marza, T. Ferreira, Is fat so bad? Modulation of endoplasmic reticulum stress by lipid droplet formation, Biol. Cell. 103 (2011) 271–285.
[12] H.P. Harding, Y. Zhang, H. Zeng, I. Novoa, P.D. Lu, M. Calfon, N. Sadri, C. Yun, B. Popko, R. Paules, D.F. Stojdl, J.C. Bell, T. Hettmann, J.M. Leiden, D. Ron, An integrated stress response regulates amino acid metabolism and resistance to oxidative stress, Mol. Cell 11 (2003) 619–633.
[13] M. Irmler, M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L.E. French, J. Tschopp, Inhibition of death receptor signals by cellular FLIP, Nature 388 (1997) 190–195.
[14] R.J. Kaufman, D. Scheuner, M. Schroder, X. Shen, K. Lee, C.Y. Liu, S.M. Arnold, The unfolded protein response in nutrient sensing and differentiation, Nat. Rev. Mol. Cell Biol. 3 (2002) 411–421.
[15] A.H. Lee, E.F. Scapa, D.E. Cohen, L.H. Glimcher, Regulation of hepatic lipogenesis by the transcription factor XBP1, Science 320 (2008) 1492–1496.
[16] J.S. Lee, R. Mendez, H.H. Heng, Z.Q. Yang, K. Zhang, Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation, Am. J. Transl. Res. 4 (2012) 102–113.
[17] S.J. Lee, J. Zhang, A.M. Choi, H.P. Kim, Mitochondrial dysfunction induces formation of lipid droplets as a generalized response to stress, Oxidative Med. Cell. Longev. 2013 (2013) 327167.
[18] S.E. Logue, P. Cleary, S. Saveljeva, A. Samali, New directions in ER stress-induced cell death, Apoptosis 18 (2013) 537–546.
[19] Y. Ma, L.M. Hendershot, The unfolding tale of the unfolded protein response, Cell 107 (2001) 827–830.
[20] S.J. Marciniak, D. Ron, Endoplasmic reticulum stress signaling in disease, Physiol. Rev. 86 (2006) 1133–1149.
[21] E.S. Marini, C. Giampietri, S. Petrungaro, S. Conti, A. Filippini, L. Scorrano, E. Ziparo, The endogenous caspase-8 inhibitor c-FLIP regulates ER morphology and crosstalk with mitochondria, Cell Death Differ. (2014) http://dx.doi.org/10.1038/cdd.2014.197.
[22] S. Martin, R.G. Parton, Lipid droplets: a unified view of a dynamic organelle, Nat. Rev. Mol. Cell Biol. 7 (2006) 373–378.
[23] R. Martin-Perez, M. Niwa, A. Lopez-Rivas, ER stress sensitizes cells to TRAIL through down-regulation of FLIP and Mcl-1 and PERK-dependent up-regulation of TRAIL-R2, Apoptosis 17 (2012) 349–363.
[24] O. Micheau, M. Thome, P. Schneider, N. Holler, J. Tschopp, D.W. Nicholson, C. Briand, M.G. Grutter, The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex, J. Biol. Chem. 277 (2002) 45162–45171.
[25] T. Okada, H. Yoshida, R. Akazawa, M. Negishi, K. Mori, Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response, Biochem. J. 366 (2002) 585–594.
[26] S. Oyadomari, H.P. Harding, Y. Zhang, M. Oyadomari, D. Ron, Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice, Cell Metab. 7 (2008) 520–532.
[27] U. Ozcan, Q. Cao, E. Yilmaz, A.H. Lee, N.N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Gorgun, L.H. Glimcher, G.S. Hotamisligil, Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes, Science 306 (2004) 457–461.
[28] G.D. Pavitt, D. Ron, New insights into translational regulation in the endoplasmic reticulum unfolded protein response, Cold Spring Harb. Perspect. Biol. 4 (2012).
[29] H.L. Ploegh, A lipid-based model for the creation of an escape hatch from the endoplasmic reticulum, Nature 448 (2007) 435–438.
[30] A. Pol, S.P. Gross, R.G. Parton, Review: biogenesis of the multifunctional lipid droplet: lipids, proteins, and sites, J. Cell Biol. 204 (2014) 635–646.
[31] A. Ruiz-Vela, C. Aguilar-Gallardo, A.M. Martinez-Arroyo, M. Soriano-Navarro, V. Ruiz, C. Simon, Specific unsaturated fatty acids enforce the transdifferentiation of human cancer cells toward adipocyte-like cells, Stem Cell Rev. 7 (2011) 898–909.

[32] D.T. Rutkowski, R.J. Kaufman, That which does not kill me makes me stronger: adapting to chronic ER stress, Trends Biochem. Sci. 32 (2007) 469–476.
[33] D.B. Savage, PPAR gamma as a metabolic regulator: insights from genomics and pharmacology, Expert Rev. Mol. Med. 7 (2005) 1–16.
[34] M. Shibata, K. Yoshimura, H. Tamura, T. Ueno, T. Nishimura, T. Inoue, M. Sasaki, M. Koike, H. Arai, E. Kominami, Y. Uchiyama, LC3, a microtubule-associated protein1A/B light chain3, is involved in cytoplasmic lipid droplet formation, Biochem. Biophys. Res. Commun. 393 (2010) 274–279.
[35] R. Singh, S. Kaushik, Y. Wang, Y. Xiang, I. Novak, M. Komatsu, K. Tanaka, A.M. Cuervo, M.J. Czaja, Autophagy regulates lipid metabolism, Nature 458 (2009) 1131–1135.
[36] R. Sriburi, S. Jackowski, K. Mori, J.W. Brewer, XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum, J. Cell Biol. 167 (2004) 35–41.
[37] D.J. Todd, A.H. Lee, L.H. Glimcher, The endoplasmic reticulum stress response in immunity and autoimmunity, Nat. Rev. Immunol. 8 (2008) 663–674.
[38] S.E. Tran, A. Meinander, T.H. Holmstrom, A. Rivero-Muller, K.M. Heiskanen, E.K. Linnau, M.J. Courtney, D.D. Mosser, L. Sistonen, J.E. Eriksson, Heat stress downregulates FLIP and sensitizes cells to Fas receptor-mediated apoptosis, Cell Death Differ. 10 (2003) 1137–1147.
[39] I. Venza, M. Visalli, R. Oteri, D. Teti, M. Venza, Class I-specific histone deacetylase inhibitor MS-275 overrides TRAIL-resistance in melanoma cells by downregulating c-FLIP, Int. Immunopharmacol. 21 (2014) 439–446.
[40] S.J. White-Gilbertson, L. Kasman, J. McKillop, T. Tirodkar, P. Lu, C. Voelkel-Johnson, Oxidative stress sensitizes bladder cancer cells to TRAIL mediated apoptosis by down-regulating anti-apoptotic proteins, J. Urol. 182 (2009) 1178–1185.
[41] C. Zhang, Y. He, M. Okutsu, L.C. Ong, Y. Jin, L. Zheng, P. Chow, S. Yu, M. Zhang, Z. Yan, Autophagy is involved in adipogenic differentiation by repressesing proteasome-dependent PPARgamma2 degradation, Am. J. Physiol. Endocrinol. Metab. 305 (2013) E530–E539.
[42] K. Zhang, R.J. Kaufman, The unfolded protein response: a stress signaling pathway critical for health and disease, Neurology 66 (2006) S102–S109.
[43] Z. Zheng, C. Zhang, K. Zhang, Measurement of ER stress response and inflammation in the mouse model of nonalcoholic fatty liver disease, Methods Enzymol. 489 (2011) 329–348.
[44] L. Shang, X. Wang, AMPK and mTOR coordinate the regulation of Ulk1 and mammalian autophagy initiation, Autophagy 7 (2011) 924–926.